Characterization and Reconstruction of Nanolipoprotein Particles (NLPs) by Cryo-EM and Image Reconstruction

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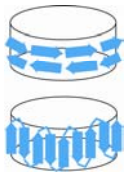
Background

Abstract

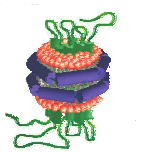
Nanolipoprotein particles (NLPs) are small 10-20 nm diameter assemblies of apolipoproteins and lipids. At Lawrence Livermore National Laboratory (LLNL), we have constructed multiple variants of these assemblies. NLPs have been generated from a variety of lipoproteins, including apolipoprotein AI, apolipophorin III, apolipoprotein E4 22K, and MSP1T2 (Nanodisc, Inc.). Lipids used included DMPC (bulk of the bilayer material), DMPE (in various amounts), and DPPC. NLPs were made in either the absence or presence of the detergent cholate. We have collected electron microscopy data as a part of the characterization component of this research. Although purified by size exclusion chromatography (SEC), samples are somewhat heterogeneous when analyzed at the nanoscale by negative stained cryo-EM. Images reveal a broad range of shape heterogeneity, suggesting variability in conformational flexibility, in fact, modeling studies point to dynamics of inter-helical loop regions within apolipoproteins as being a possible source for observed variation in NLP size. Initial attempts at three-dimensional reconstructions have proven to be challenging due to this size and shape disparity. We are pursuing a strategy of computational size exclusion to group particles into subpopulations based on average particle diameter. We show here results from our ongoing efforts at statistically and computationally subdividing NLP populations to realize greater homogeneity and then generate 3D reconstructions.

Introduction

The assembly and function of lipoprotein particles have been studied since they were first isolated from human plasma in the 1920s (1). More recently, attention has focused on plasma lipoproteins and the correlation between cholesterol levels and coronary heart disease (CAD), especially the inverse correlation between the concentration of high density lipoprotein (HDL) and CAD. The latter has led to molecular level investigations of protein-lipid interactions, including detailed studies focusing on reconstitution of HDL-like particles. Apolipoproteins (A-1, E and C) were shown to associate with phospholipids in vitro, often forming discoidal-shaped complexes similar to nascent HDL particles produced by the liver (2, 3). Studying the self-assembly of these particles in vitro was initiated in the early 1980's when Jonas and coworkers described the association of lipid molecules with purified apolipoprotein A-1 (4, 5). Recently, Sligar and coworkers reported that a number of recombinantly derived ApoAI variants self-assemble in the presence of phospholipid, forming discoidal particles; they refer to these particles as nanodiscs (6). All of the recombinant apoA-1 variants, some larger than the native protein and some smaller, are reported to form nanodisc-like structures (7). Working with recombinant Apolipoprotein E variants (most notably an N-terminal 22 KDa fragment, E422K), Weisgraber and coworkers also report the formation of discoidal particles. Two independent groups have also reported similar type particles being formed using apolipoproteins from insect flight muscles combined with phospholipids (8, 9).

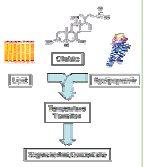


NLP "belt" and "picket-fence" models



Cartoon model of NLP containing membrane protein

Materials and Methods



Schematic of disc assembly

NLP assembly and production

Phospholipid was solubilized either by sonication in TBS to produce small unilamellar vesicles (SUVs) or by rehydration of chloroform/nitrogen deposited film with TBS and cholate. Chloroform was removed using a gentle stream of nitrogen gas and TBS buffer was added to resolubilize/suspend phospholipid; cholate concentration is 20 mM (if included). Once lipid was solubilized, apolipoprotein was introduced to begin the self-assembly process. Protein and lipid assemblies were cycled through three transitions of 30 C and 20 C, followed by 23.8 C overnight. Following assembly, correctly sized NLPs were separated from larger lipid-dominant structures and smaller protein-dominant structures using size exclusion chromatography over Sephadex200 column (GE Healthcare). Following SEC, NLP fractions were concentrated for characterization.

Materials and Methods

Sample preparation and data collection

Samples of NLP prep LD54 (DMPC and lipophorin III/*B. mori*, 158:1(mol/mol) lipid to scaffold protein) were diluted using TBS to achieve a final concentration of approximately 1.0 mg/ml. Three µl of each sample were pipetted onto a carbon coated 400 mesh copper EM grid (Quantifoil). Sample prep includes 2% ammonium molybdate to improve sample contrast. After sitting for 1 minute, the sample was blotted with Whatman filter paper and flash frozen by plunging into liquid ethane. 32 micrograph images were recorded using a JEOL 2100 FEG with a Teitz digital CCD(UC Davis) at 200 keV as 16-bit tiffs at varying magnifications.

Image processing and 3D reconstruction

Single particle image reconstructions were conducted using EMAN. At present, EMAN is compiled and operated on two parallel supercomputing cluster machines at LLNL, Zeus, and Thunder:

•**Zeus** is a 288 node (8 cpu/node), 11 Tflop/sec machine, utilizing InfiniBand interconnects and running the CHAOS 3.2 Linux OS.
•**Thunder** is a 1024 node (4 cpu/node), 23 Tflop/sec machine, utilizing Quadrics Elan 4 switch interconnects and running CHAOS 3.1 Linux OS.

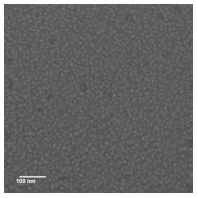
Thunder parallel cluster

Zeus parallel cluster

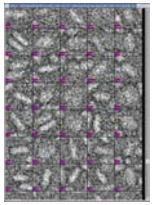
Results

Cryo-stained electron micrographs

NLPs were made using the cholate-free method and negative stained cryo-EM images show no differences in overall structure of the discs. Clearly, the NLPs show polydispersity in structure and size. However, no roulouax are observed in these preparations, minimizing the possibility that the discoidal shape is due to staining artifacts as was previously postulated (10). Although the field is crowded, discoidal shaped structures are obvious throughout the sample. Particle sizes range from 10-12 nm, ± 2 nm, scale bar of 100 nm.



Cryo-electron micrograph of empty NLPs, magnification = 60,000x

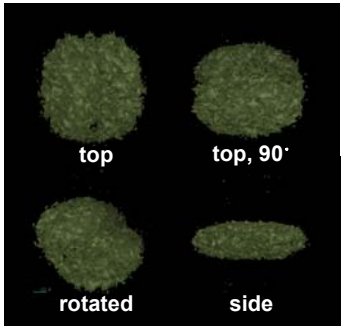


Boxed NLP particle views

Results

Three dimensional reconstructions

128x128 pixel images were boxed from the starting micrograph using the program BOXER. From the micrograph, 1377 particles were boxed. During the reconstruction attempts no symmetry was enforced as the NLP particles in the micrograph appeared to be polydisperse. Initial reconstructions verified that the sample was heterogeneous and that additional pre-processing steps are required to produce an adequate initial model before attempting to perform reconstructions using all 32 micrographs. Visualizations pictured here were performed using Chimera (UCSF).



Preliminary 3D models

Conclusions

We have conducted an initial pass at determining the nanoscale characterization of nanolipoprotein particles or NLPs utilizing cryo-electron microscopy and three-dimensional image processing. Although purified by size exclusion chromatography to a somewhat homogeneous size population, these NLPs are actually quite polydisperse on the nanometer scale and represent a challenge with respect to single-particle image processing. NLPs may provide a platform for which further research can use to characterize membranes, apolipoproteins, drug delivery, and membrane proteins within the NLP structures. Future plans for the 3D reconstruction methodology are to implement statistical sub-categorization of NLP populations for attempts at more successful reconstructions. If a better subpopulation is obtained, we may be able to make a more accurate determination of the belt vs. picket-fence model, in addition to providing structural detail about any incorporated membrane proteins.

Acknowledgements

We gratefully acknowledge support from Laboratory Directed Research and Development (LDRD) grants 06-LW-064 and 06-SI-003. We further acknowledge computational resources from the Livermore Computing (LC).

References

1. Machebeouf M. Bull. Soc. Chim. Biol. 1929;11:268.
2. Atkinson D, Small DM. Recombinant lipoproteins: implications for structure and assembly of native lipoproteins. Annu Rev Biophys Biophys Chem 1986;15:403-56.
3. Jonas A. Reconstitution of high-density lipoproteins. Methods Enzymol 1986;128:553-82.
4. Jonas A, Drengler SM, Patterson BW. Two types of complexes formed by the interaction of apolipoprotein A-I with vesicles of L-alpha-dimyristoylphosphatidylcholine. J Biol Chem 1990;265(5):2183-9.
5. Wald JH, Krul ES, Jonas A. Structure of apolipoprotein A-I in three homogeneous, reconstituted high density lipoprotein particles. J Biol Chem 1990;265(32):20037-43.
6. Bayburt TH, Grinkova, Y. V. and Sligar, S. G. Self-Assembly of Discoidal Phospholipid Bilayer Nanoparticles with Membrane Scaffold Proteins. Nano Lett 2002;2:853-856.
7. Denisov IG, Grinkova YV, Lazarides AA, Sligar SG. Directed self-assembly of monodisperse phospholipid bilayer Nanodiscs with controlled size. J Am Chem Soc 2004;126(11):3477-87.
8. Ryan RO, Schieve D, Wientzek M, Narayanaswami V, Oikawa K, Kay CM, et al. Bacterial expression and site-directed mutagenesis of a functional recombinant apolipoprotein. J Lipid Res 1995;36(5):1066-72.
9. Niere M, Mekalanos C, Dettloff M, Weise C, Ziegler M, Wiesner A. Insect immune activation by recombinant Galleria mellonella apolipophorin III(1). Biochim Biophys Acta 1998;1433(1-2):16-26.
10. Carlson JW, Jonas A, Sligar SG. Imaging and manipulation of high-density lipoproteins. Biophys J 1997;73(3):1184-9.

*This work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract No. W-7405-Eng-48.

UCRL-CONF-232062